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Ezetimibe potently inhibits cholesterol absorption but does not affect acute hepatic or intestinal cholesterol synthesis in rats

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1 Ezetimibe (1-(4-fluorophenyl)-(3R)-[3-(4-fluorophenyl)-(3S)-hydroxypropyl]-(4S)-(4-hydroxyphenyl)-2-azetidinone) and its analog SCH48461 are potent and selective cholesterol absorption inhibitors that inhibit the transport of cholesterol across the intestinal wall, thereby lowering plasma cholesterol.

2 After a dose response for ezetimibe in rats was established, experiments were conducted to determine whether acute administration could alter hepatic or intestinal cholesterol synthesis. To determine whether this class of intestinal cholesterol absorption inhibitors could discriminate between newly synthesized cholesterol in the intestine *versus* exogenously administered cholesterol, rats were intraduodenally dosed with ^{14}C-cholesterol and ^{3}H-mevalonate, and mesenteric lymph was analyzed for radiolabeled cholesterol and cholesteryl ester content.

3 Ezetimibe attenuated diet-induced hypercholesterolemia 60–94% at doses of 0.1–3 mg kg^{-1} in rats. A single administration of ezetimibe did not have a direct effect on intestinal or hepatic cholesterol synthesis, while ketoconazole significantly inhibited cholesterol synthesis after a single dose. The ezetimibe analog, SCH48461, inhibited the movement of exogenously administered cholesterol into lymph, but did not affect the appearance of newly synthesized cholesterol into lymph.

4 These data suggest that this class of cholesterol absorption inhibitors does discriminate by blocking the movement of exogenous cholesterol in the enterocyte before it reaches the intracellular cholesterol pool to be incorporated into intestinal lipoproteins, without affecting the incorporation of newly synthesized cholesterol into intestinal lipoproteins.

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Keywords: Cholesterol absorption inhibitor; hepatic cholesterol; intestinal cholesterol; lymph; rat; cholesteryl ester; ezetimibe

Abbreviations: AAALAC, American Association for Accreditation of Laboratory Animal Care; DPBS, Dulbecco's phosphate-buffered saline; ED_{50}, effective dose at which 50% inhibition occurs; HDL, high-density lipoprotein; HMG, hydroxylmethylglutaryl; HPLC, high-performance liquid chromatography; LDL, low-density lipoprotein; SEM, standard error of the mean

Introduction

We have previously described the discovery of ezetimibe (SCH58235) and one of its analogs (SCH48461; Figure 1), which are members of a novel class of intestinal cholesterol absorption inhibitors. In preclinical models, this new class of compounds reduced diet-induced hypercholesterolemia in mice, hamsters, rats, rabbits, dogs, and monkeys (Salisbury *et al.*, 1995; van Heek *et al.*, 1997; 2000; 2001b; Davis *et al.*, 2001b). Ezetimibe inhibits the intestinal absorption of cholesterol without affecting the absorption of triglycerides, bile acids, or fat-soluble vitamins (van Heek *et al.*, 2001c). In a hamster model of combined hyperlipidemia, ezetimibe significantly reduced both plasma cholesterol and triglyceride (van Heek *et al.*, 2001a). Ezetimibe completely ablated the development of atherosclerosis in apo E knockout mice (Davis *et al.*, 2001a). Ezetimibe given in combination with hydroxylmethylglutaryl (HMG) CoA reductase inhibitors ('statins'), which inhibit cholesterol synthesis, reduced plasma cholesterol in dogs in either an additive or synergistic manner, depending on the statin employed (Davis *et al.*, 2001b). In hypercholesterolemic humans, ezetimibe given as monotherapy lowered low-density lipoprotein (LDL) cholesterol, raised high-density lipoprotein (HDL) cholesterol, and modestly lowered plasma triglyceride (Bays *et al.*, 2001). In combination with low doses of statins, ezetimibe lowered LDL cholesterol as much as 60% in 2 weeks of treatment in hypercholesterolemic patients (Kosoglou *et al.*, 2002). In homozygous familial hypercholesterolemia, the combination of ezetimibe with a statin lowered plasma cholesterol more than was previously possible with other pharmacotherapy (Gagne *et al.*, 2002). Lastly, ezetimibe alone effectively reduced serum plant sterols in patients with sitosterolemia (von Bergmann *et al.*, 2002). Phase III trials for ezetimibe, alone and in combination with statins, have been completed, and in addition to demonstrating efficacy, ezetimibe has been shown to be well tolerated (Gagne *et al.*, 2002; Stein *et al.*, 2002; Vermaak *et al.*, 2002; von Bergmann *et al.*, 2002). Ezetimibe was launched in late 2002.

Physiologically, ezetimibe prevents the transport of dietary and biliary cholesterol, and presumably plant sterols (von Bergmann *et al.*, 2002), across the intestinal wall (van Heek

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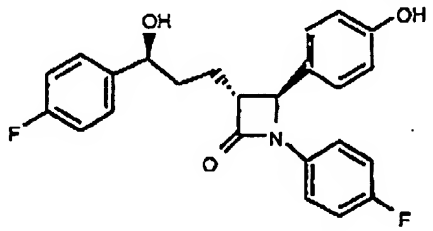
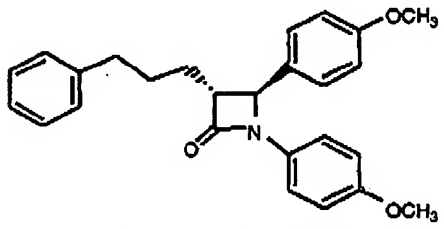


Ezetimibe (SCH 58235)



Ezetimibe analog (SCH 48461)

Figure 1 Structure of ezetimibe (SCH58235) and the ezetimibe analog, SCH48461. Ezetimibe: 1-(4-fluorophenyl)-3(R)-[3-(4-fluorophenyl)-3(S)-hydroxypropyl]-4(S)-(4-hydroxyphenyl)-2-azetidinone. SCH48461: (3R,4S)-1,4-*bis*-(4-methoxyphenyl)-3-(3-phenylpropyl)-2-azetidinone.

et al., 2000). Despite all that is known about the efficacy of ezetimibe in preclinical models and human clinical trials, the molecular mechanism by which ezetimibe inhibits cholesterol absorption is not understood. Indeed, the process by which cholesterol is absorbed and processed in the intestine is poorly understood and has been a subject of great interest for decades (for review, see Homan & Krause, 1997; Dawson & Rudel, 1999; Turley, 1999; Hussain, 2000). The present work was undertaken to determine whether ezetimibe could acutely affect the process of cholesterol synthesis in either the liver or the intestine. In addition, studies were conducted to determine whether this class of cholesterol absorption inhibitors could discriminate between cholesterol that was exogenously administered into the intestinal lumen *versus* cholesterol that was newly synthesized in the enterocyte.

Methods

Dose response of ezetimibe in diet-induced hypercholesterolemic rats

Male Sprague–Dawley rats (250–300 g) were acclimated to a 12 : 12 light : dark cycle and allowed chow and water *ad libitum*. Rats were then grouped into five groups of six based on body weight. All rats were placed on a 1% cholesterol/0.5% cholic acid diet (Research Diets; New Brunswick, NJ, U.S.A.) for 7 days and orally gavaged daily with either vehicle alone (0.5 ml corn oil), or 0.1, 0.3, 1.0, or 3.0 mg kg^{-1} of ezetimibe in corn oil. After 7 days, animals were euthanized after anesthesia, blood was collected, and plasma was separated by low-speed centrifugation at 4°C for cholesterol analysis (Wako; Osaka, Japan). The value for plasma cholesterol in rats under chow-fed conditions shown in Figure 2 is a historical average collected from 100 rats (200–250 g) and is included in the

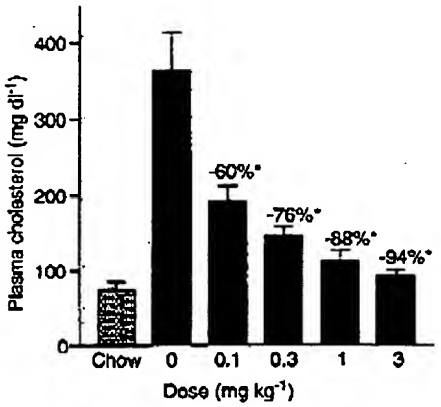


Figure 2 Dose response of ezetimibe in diet-induced hypercholesterolemia in rats. Rats were fed a 1% cholesterol/0.5% cholic acid diet for 7 days and were daily gavaged with either corn oil alone or corn oil containing ezetimibe (0.1–3 mg kg^{-1}). The gray bar represents the plasma cholesterol of rats ($n=100$) maintained on chow for comparison. Values are mean ± standard error of the mean (s.e.m.); $n=6$/group. *$P<0.05$ compared to cholesterol-fed control group.

graph as a baseline benchmark for comparison with the cholesterol-fed rats.

Effect of a single administration of ezetimibe on hepatic and intestinal cholesterol synthesis

Fasted male Sprague–Dawley rats (250–300 g), which had been on normal chow diet, were anesthesized with Inactin (100 mg kg^{-1} i.p.; Abbott Labs; Chicago, IL, U.S.A.). All rats were fitted with tracheal tubes and were intraduodenally cannulated as previously described (van Heek *et al.*, 1997). At $t=0$, rats ($n=5$/group) were given either vehicle (1 ml donor rat bile), ezetimibe (0.03 and 10 mg kg^{-1} in bile), or ketoconazole (50 mg kg^{-1} in bile) intraduodenally. Bile was chosen as the vehicle because it was very effective at solubilizing drugs for direct intraduodenal delivery as previously described (van Heek *et al.*, 1997; 2000). The doses of ezetimibe used in these experiments were the approximated ED_{50} (effective dose at which 50% inhibition occurs, 0.03 mg kg^{-1}) and a dose greater than the ED_{100} (10 mg kg^{-1}). The dose of ketoconazole (50 mg kg^{-1}) was reported as the effective dose to interfere with cholesterol synthesis (Princen *et al.*, 1986). At $t=1$ h, 10 μCi of ^{3}H-mevalonate in sodium taurocholate (19 mM in Dulbecco's phosphate-buffered saline (DPBS, pH 6.4) was delivered intraduodenally to each rat. Rats were killed at $t=2.5$ h. Intestines and livers were collected and rinsed with saline. The tissues were saponified overnight at 55°C in a 40% ethanolic potassium hydroxide solution and were extracted three times with 6 ml of petroleum ether. Cholesterol and cholesterol precursors in the extracts from livers were separated by high-performance liquid chromatography (HPLC); 0.020 ml aliquots were injected onto a Zorbex CN column (Dupont; Wilmington, DE, U.S.A.) and eluted with 99.9% hexane : 0.1% isopropanol at a rate of 1 ml min^{-1}. Squalene, lanosterol, cholesterol, desmosterol, lathosterol, and dehydrocholesterol (Supelco; Bellefonte, PA, U.S.A.) were used as HPLC standards. A total of 96 fractions were collected into a 96-well Wallac

microliter plate at 0.26 min intervals. The solvent was allowed to evaporate after which 0.300 ml of Optiphase scintillation cocktail was added to each well. Total ^{3}H radioactivity was determined using a Wallac Microbeta Counter. Extracts from intestine could not be analyzed by HPLC because of insufficient radioactivity in 0.020 ml of the sample, the maximum volume allowed for this automated HPLC method. Therefore, 0.05–0.10 ml of extract was separated by thin layer chromatography in a two-solvent system (solvent system 1:97:100:3 petroleum ether:diethyl ether:acetic acid; solvent system 2:194:6 petroleum ether:diethyl ether; Bergstrom *et al.*, 1993). Fractions of 1 cm from the origin to solvent front were scraped and analyzed for radioactivity. Fractions 6–16 were identical in all groups; therefore, only dpm in the cholesterol fraction (fraction 2) and the lanosterol fraction (fraction 3) are reported.

Effect of the ezetimibe analog (SCH48461) on absorption, synthesis, and secretion of cholesterol and cholesteryl ester into the lymph of rats

Fasted male Sprague–Dawley rats (250–300 g), which had been on normal chow diet, were orally gavaged with corn oil vehicle (0.5 ml; $n=4$) or SCH48461 in corn oil ($n=7$; 10 mg kg^{-1}) 3 h prior to surgery. Previous experience in our laboratory demonstrated that gavaging corn oil several hours prior to lymph collection improved visibility for lymph duct cannulation, as well as increased lymph flow and output. Rats were anesthesized with Inactin (100 mg kg^{-1} i.p.), were fitted with tracheal tubes, and were intraduodenally cannulated as previously described (van Heek *et al.*, 1997). The main mesenteric lymph vessel was cannulated. An emulsion containing triolein (35.4 mg per dose; Sigma; St Louis, MO, U.S.A.), L-α-phosphatidylcholine (6.69 mg per dose; Sigma), sodium taurocholate (3 ml per dose; 19 mM in DPBS, pH 6.4; Sigma), 1 μCi ^{14}C-cholesterol and 10 μCi ^{3}H-mevalonate (NEN; Boston, MA, U.S.A.) was placed directly into the intestinal lumen via the catheter and the mesenteric lymph was collected for 4 h. After measurement of the volume of lymph collected and lipid extraction of the lymph, cholesterol and cholesteryl esters were separated by thin layer chromatography and were analyzed for ^{3}H and ^{14}C radioactivity.

Statistical analyses

Statistical significance in the dose response was determined by analysis of variance, followed by Student's *t*-test. Statistical differences between control and treated groups in all other experiments were tested using Student's *t*-test.

American Association for Accreditation of Laboratory Animal Care (AAALAC) Statement

All studies were conducted in an AAALAC accredited facility following protocols approved by the Schering-Plough Research Institute's Animal Care and Use Committee. The procedures were performed in accordance with the principles and guidelines established by the NIH for the care and use of laboratory animals.

Results

Chemical structures

The chemical structures of ezetimibe (SCH58235) and the ezetimibe analog (SCH48461) are shown in Figure 1. SCH48461 was the first cholesterol absorption inhibitor that went into clinical trials. Ezetimibe was discovered through the identification of the active metabolites of SCH48461 and was found to be 400 times more potent than SCH48461 in the hypercholesterolemic rhesus monkey (van Heek *et al.*, 1997). Subsequently, ezetimibe replaced SCH48461 and has proceeded through completion of Phase III clinical trials.

Dose response of ezetimibe in diet-induced hypercholesterolemic rats

Rats that were fed a 1% cholesterol/0.5% cholic acid diet for 7 days developed hypercholesterolemia that was approximately five times the level of plasma cholesterol in chow-fed controls. Once per day oral dosing of ezetimibe (0.1–3 mg kg^{-1}) led to a dose-dependent decrease (60–94%) in diet-induced hypercholesterolemia (Figure 2).

Effect of a single administration of ezetimibe on hepatic and intestinal cholesterol synthesis

This experiment was performed to assess the acute effect of a pharmacological (0.03 mg kg^{-1}) and a high dose (10 mg kg^{-1}) of ezetimibe on the synthesis of cholesterol and its precursors in the liver and intestine of rats. Ketoconazole, an inhibitor of lanosterol demethylase, was included as a positive control. Rats were given a single oral gavage of vehicle, ezetimibe (0.030, 10 mg kg^{-1}) or ketoconazole (50 mg kg^{-1}). In the HPLC chromatograms of the liver extracts (Figure 3), the first peak represents squalene, the second lanosterol, and the third cholesterol with possible minor amounts of desmosterol, lathosterol, and dehydrocholesterol. Compared to control (Figure 3a), neither the ED_{50} dose (0.03 mg kg^{-1}; Figure 3b) nor the high dose (10 mg kg^{-1}; Figure 3c) of ezetimibe had an effect on the synthesis of cholesterol or its precursors from ^{3}H-mevalonate. Ketoconazole did cause an accumulation of lanosterol as expected (total dpm significantly different at $P<0.0001$ compared to lanosterol in the control group; Figure 3d). These data were mimicked in the intestinal extracts (Figure 4): synthesis of cholesterol and lanosterol was not affected by ezetimibe at either the low or high dose, yet there was an accumulation of lanosterol ($P<0.02$ compared to control) in the intestines of rats treated with ketoconazole.

Effect of the ezetimibe analog (SCH48461) on absorption, synthesis, and secretion of cholesterol and cholesteryl ester into the lymph of rats

After oral gavage of corn oil vehicle or 10 mg kg^{-1} of the ezetimibe analog, SCH48461, intraduodenal and lymph duct-cannulated rats were given an emulsion containing both ^{14}C-cholesterol and ^{3}H-mevalonate, a cholesterol synthesis precursor. Lymph was then collected for 4 h, and the amount of ^{14}C-free cholesterol, ^{14}C-cholesteryl ester, ^{3}H-free cholesterol, and ^{3}H-cholesteryl ester radioactivity in the collected lymph was determined as described in Methods. The volume of lymph

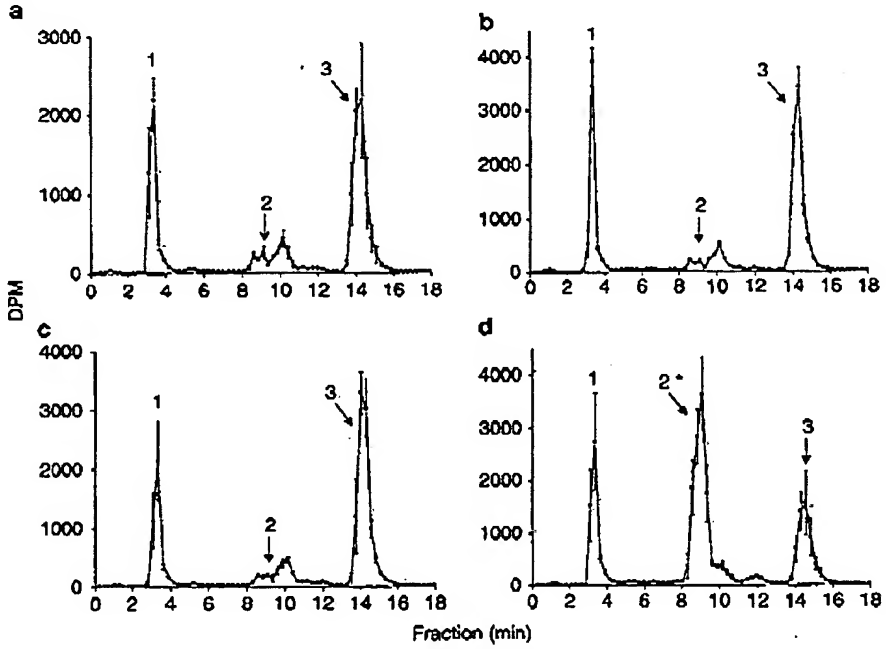


Figure 3 Effect of ezetimibe and ketoconazole on the hepatic synthesis of cholesterol from ^{3}H-mevalonate in the rat: HPLC chromatograms of ^{3}H-cholesterol and its precursors. (a) Vehicle, (b) ezetimibe (0.03 mg kg^{-1}), (c) ezetimibe (10 mg kg^{-1}), (d) ketoconazole (50 mg kg^{-1}). The first peak (Peak 1; 4 min) represents squalene, the second (Peak 2; 8 – 11 min) is lanosterol, and the third (Peak 3; 15 min) is cholesterol with possible minor amounts of desmosterol, lathosterol, and dehydrocholesterol. Values are mean±s.e.m.; $n=5$ per group. *$P<0.05$; total dpm in the peak significantly different from the same peak in the control group.

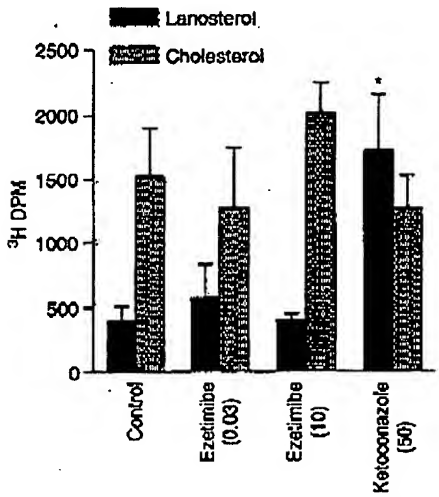


Figure 4 Effect of ezetimibe and ketoconazole on the intestinal synthesis of cholesterol from ^{3}H-mevalonate in the rat. Intestines were extracted as described in Methods and separated by thin layer chromatography; Fractions of 1 cm were scraped and analyzed for radioactivity. Only dpm for cholesterol (fraction 2) and lanosterol (fraction 3) are shown since all other fractions were identical between the treatments. Values are mean±s.e.m., $n=5$ per group. *$P<0.05$ compared to control.

collected over 4 h did not differ between control and SCH48461-treated rats (1.98±0.06 *versus* 1.87±0.29 ml). The appearance of ^{14}C-free cholesterol and ^{14}C-cholesteryl ester in lymph after intraduodenal dosing of ^{14}C-free cholesterol was significantly diminished by the ezetimibe analog by 74 and 97%, respectively (Figure 5a). In contrast, the appearance in lymph of newly synthesized ^{3}H-free cholesterol and ^{3}H-cholesteryl ester from ^{3}H-mevalonate was equivalent in the absence and presence of the ezetimibe analog (Figure 5b).

Discussion

The present studies demonstrate that ezetimibe potently diminishes diet-induced hypercholesterolemia in rats. This is likely not because of a direct effect of ezetimibe on hepatic or intestinal cholesterol synthesis, since an acute administration of a pharmacological (0.03 mg kg^{-1}) or a high dose (10 mg kg^{-1}) of ezetimibe had no effect on synthesis in either organ. However, an indirect increase in cholesterol synthesis after multiple dosing of this class of cholesterol absorption inhibitors is quite likely since an increase in hepatic HMG CoA reductase activity has been observed after chronic treatment (Davis *et al.*, 2001b). To determine whether this class of cholesterol absorption inhibitors could discriminate in the intestine between exogenously administered cholesterol *versus* newly synthesized cholesterol, radiolabeled cholesterol and a radiolabeled cholesterol precursor, mevalonate, were simultaneously administered to the intestines of lymph cannulated rats. This experiment demonstrated that the ezetimibe analog, SCH48461, could prevent the transport of exogenously administered radiolabeled cholesterol from the intestinal lumen, through the intestinal wall, and into the

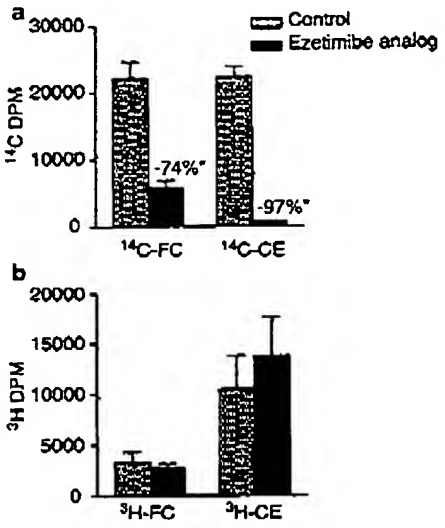


Figure 5 Effect of the ezetimibe analog (SCH48461) on the absorption, synthesis, and secretion of radiolabeled cholesterol and cholesteryl ester into lymph of rats. (a) Appearance in lymph of ^{14}C-free cholesterol (FC) and ^{14}C-cholesteryl (CE) ester after ^{14}C-free cholesterol was administered to the intestinal lumen. (b) Appearance in lymph of newly synthesized 3H-free cholesterol and ^{3}H-cholesteryl ester after ^{3}H-mevalonate was administered to the intestinal lumen. Each rat was given both ^{14}C-free cholesterol and ^{3}H-mevalonate so that cholesterol absorption and cholesterol synthesis could be studied under identical conditions. Values are mean±s.e.m., $n=4$ (control) and 7 (SCH48461; 10 mg kg^{-1}) per group. *$P<0.0001$ compared to control.

lymph. Yet, the ezetimibe analog had no effect on the appearance of newly synthesized cholesterol in lymph.

Since the molecular mechanism of ezetimibe is not known, the present findings may help to elucidate steps in the cholesterol metabolism pathway affected by these cholesterol absorption inhibitors. In general, intestinal cholesterol absorption begins with the micellar solubilization of both dietary and biliary cholesterol in the lumen of the intestine. The cholesterol is then transferred from the micelles to the surface membrane of the enterocyte, and into the cytoplasmic compartment. Cholesterol moves to the endoplasmic reticulum where it may be esterified by ACAT to form cholesteryl ester. Free cholesterol and cholesteryl esters are packaged into chylomicrons, which are then secreted into the lymph. For excellent detailed reviews of the steps of cholesterol absorption, trafficking, and incorporation into chylomicrons, please see Dawson & Rudel (1999), Turley (1999) and Hussain (2000). Despite what is known about cholesterol absorption, it is generally agreed upon that the steps involved in cholesterol absorption between the micellar solubilization step and the cholesterol esterification step are poorly understood. If it is assumed that absorbed cholesterol and newly synthesized cholesterol become part of the same intracellular cholesterol pool in the enterocyte prior to ACAT esterification and subsequent incorporation into chylomicrons, then the *in vivo* data presented here suggest that this class of cholesterol absorption inhibitors is active at a step in the cholesterol absorption process prior to entry into the intracellular cholesterol pool. Otherwise, one would have expected the ezetimibe analog to decrease the appearance of both exogenous and newly synthesized cholesterol and cholesteryl ester in lymph. These data support the conclusions of others (Detmers *et al*., 2000; Hernandez *et al*., 2000). Using different techniques from the present studies, these investigators demonstrated that a structurally different cholesterol absorption inhibitor for which the molecular mechanism is also not known, bound specifically to the brush border membrane of hamster enterocytes after oral gavage. In addition, this binding could be competed by other cholesterol absorption inhibitors, including one that is structurally similar to ezetimibe.

In the present studies, we did not attempt to separate lipoprotein classes in the lymph collected, or study other components in the lymph, because we were primarily interested in whether these cholesterol absorption inhibitors could discriminate between exogenous cholesterol and newly synthesized cholesterol. However, lymph volume output was identical in control *versus* treated animals, which suggests that a gross inhibition of lymph production does not occur in the presence of the cholesterol absorption inhibitor. Previous experiments, in which the composition of plasma postprandial lipoproteins from primates were analyzed, indicated that a single dose of the ezetimibe analog markedly reduced the free cholesterol and cholesteryl ester content of the postprandial lipoproteins, without affecting triglyceride or apoB$_{48}$ content (van Heek *et al*., 2001b). Taken together, these data indicate that ezetimibe and its analog do not directly affect cholesterol synthesis, nor do they affect the appearance of newly synthesized cholesterol in lymph lipoproteins. Yet, they potently inhibit the incorporation of exogenous cholesterol, which would include dietary and intestinal cholesterol derived from bile and cell turnover, into lymph, ultimately resulting in a significant decrease in diet-induced hypercholesterolemia.

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